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The contribution of simple random sampling to observed variations in faecal egg counts

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Abstract

It has been over 100 years since the classical paper published by Gosset in 1907, under the pseudonym “Student”, demonstrated that yeast cells suspended in a fluid and measured by a haemocytometer conformed to a Poisson process. Similarly parasite eggs in a faecal suspension also conform to a Poisson process. Despite this there are common misconceptions how to analyse or interpret observations from the McMaster or similar quantitative parasitic diagnostic techniques, widely used for evaluating parasite eggs in faeces. The McMaster technique can easily be shown from a theoretical perspective to give variable results that inevitably arise from the random distribution of parasite eggs in a well mixed faecal sample. The Poisson processes that lead to this variability are described and illustrative examples of the potentially large confidence intervals that can arise from observed faecal eggs counts that are calculated from the observations on a McMaster slide. Attempts to modify the McMaster technique, or indeed other quantitative techniques, to ensure uniform egg counts are doomed to failure and bely the ignorance of the Poisson processes. A simple method to immediately identify excess variation/poor sampling from replicate counts is provided.

Keyword: McMaster slide, FLOTAC, variability, Poisson distribution, aggregation.

1. Introduction

The McMaster technique is a widely used technique for the analysis of faecal eggs counts. Nevertheless, there appears to be widespread misconceptions about the processes that lead to variability in the observed eggs counts using this technique and how this relates to the random count distribution known as the Poisson distribution. The Poisson distribution is a discrete probability distribution that expresses the probability of a given number of events occurring in a fixed interval of time, space or volume if these events occur with a known average rate and independently of the time, space or volume since the last event.

Processes which generate Poisson distributed observations have been known for some considerable time. Indeed one of the classical works by the statistician W. S. Gosset, publishing under the well known synonym "Student" was to examine the distribution of yeast cells suspended in fluid using a haemocytometer (Student, 1907). The conclusion that Gosset reached was that random distribution of yeast cells in a fluid suspension is a Poisson process.

With the McMasters technique the sample of faeces is first mixed with an appropriate flotation solution. The suspension may then be partially filtered to remove large debris, centrifuged and re-suspended to aid visualisation and the diluted faeces are then observed on a McMasters slide. The eggs float and can be seen lying below a grid and counted. Following counting of the eggs on the slide, the numbers of eggs per gramme of faeces (epg) can be calculated by multiplying the numbers of eggs observed by an appropriate factor which depends on the dilution factor of the faeces with the flotation solution and the volume of the McMaster chamber. Although this is the basic description of the technique, there are many minor variations undertaken in different laboratories. Some of these variations are described in Pereckiene et al. (2008) and Vadlejch et al. (2011). Counting parasite eggs in a McMaster chamber is analogous to that of counting yeast cells in a haemocytometer as described by Gosset in that eggs in a faecal suspension will be randomly distributed in the same way that yeast cells are randomly distributed in a fluid suspension. Thus the random distribution of eggs in a faecal sample or diluted faecal suspension will conform to a Poisson process, provided that the sample is well mixed.

2. Variability in egg counts and random processes

Lack of understanding with regard to the variability of the McMaster technique can be illustrated by two recent papers. In Vidyashanker et al. (2012), unsuccessful attempts were made experimentally to obtain samples with uniform epgs from the same faecal sample by repeating and varying the stirring method of the faecal sample. Such an experiment was an exercise in futility;

because of Poisson processes. Levecke et al. (2011) undertook a study comparing variations on the McMaster technique and the FLOTAC (Cringoli et al., 2010) technique to demonstrate that precision increases when analytical sensitivity increases. As we discuss below, the results of Leveke et al. (2011) are entirely predictable, again because of Poisson processes. Both these manuscripts therefore, illustrate misconceptions of the distributions of eggs in a given faecal sample and how this can be described statistically.

In a well mixed faecal sample, the parasitic elements will have a random distribution in the same way that yeast cells will be randomly distributed in a well mixed fluid suspension. This is a classic Poisson process. However in the McMaster technique the variance of epg estimates between repeated samples of the same faecal sample is inflated due to the multiplication factor when transforming the raw counts to the epg. Additional errors may also arise in the laboratory (such as measurement errors for the weight of faeces or volume of diluting fluid) but these laboratory errors will not be considered further in this manuscript

Depending on the exact variation of the McMaster technique used, this arithmetical manipulation could be a multiplicative factor of 67, 50, 25, 20, 10 or some other figure. This results in a transformation of the original raw count data and its distribution into something else. As egg counting using a McMaster chamber is a Poisson process, the raw data from repeated samples from the same well mixed faecal sample are unlikely to yield the same result. Rather the results will be variable and will fit a Poisson distribution. Indeed if the raw counts of the parasitic elements from repeat samples of the same faecal sample do not follow a Poisson distribution then that is evidence that the sample was not adequately mixed before processing (Schnyder et al., 2011) rather than some profound biological process. However the calculated epg will not be Poisson distributed because the multiplication factor inflates the variance between samples.

Simple statistical theory states that if each observation from a sample with mean μ and variance γ is multiplied by a constant n, then the mean of the new sample will be $n\mu$ and the new variance will be $n^2\gamma$. This can be applied to the raw untransformed egg μ_1 counts that are Poisson distributed. These transformed counts are multiplied by the dilution factor n_1 to obtain a sample estimate of epg. Thus:

$$epg = n_1\mu_1$$

Therefore the variance of the epg, given the variance of the raw count γ_1,

$= n_1^2 \gamma_1$ (or $n_1^2 \mu_1$ as the mean of a Poisson distribution is equal to its variance)

Immediately it can be seen that the variance of the epg, $n_1^2 \mu_1$, is not equal to the mean $n_1 \mu_1$ and the transformed data is no longer Poisson distributed. Understanding this will illustrate why the observed epgs in repeated sampling from the SAME faecal sample might appear to be highly variable. As an example, it is possible to examine repeated samples from a single large well mixed faecal sample that has an epg of 200. Using a technique that has a multiplication factor of 50, the expected number of observed eggs on a McMaster slide would be a count of 4. However although this is the expected count an actual count of 4 is observed relatively infrequently. For example if 10 independent samples were taken from this faecal sample the actual observed counts could be 2, 0, 6, 3, 2, 7, 4, 3, 4, 7. These counts were generated randomly from a Poisson distribution with mean 4. The mean count of this sample is 3.8 with a variance of 5.3. The variance is of a similar magnitude as the mean and hence it can easily be shown that this random sample of 10 values conforms to a Poisson distribution of mean 4. If each is multiplied by the dilution factor of 50, the observed calculated epgs of the 10 samples are: 100, 0, 300, 150, 100, 350, 200, 150, 200, 350. This has a mean epg of 190, but the variance is now 13222. The new variance is related to the variance of the raw counts by a factor of 2500, or the dilution factor squared. Now the variance is much higher than the mean and the calculated epgs are NOT Poisson distributed. This extra Poisson variance is entirely due to the dilution factor. It should also be noted that although the expected epgs is 200, the series of 10 samples varies between 0 and 350 epg. This apparent high variability is entirely due to variability inherent in random processes that has been inflated by the dilution factor.

3. Estimation of Confidence Intervals of epgs

It is also possible to construct confidence limits of single epg calculations from the McMaster technique utilizing the Poisson distribution. An easy example is when an epg of 100 is calculated from an observed count of 2 eggs on a McMaster slide: using a dilution factor of 50 to transform the raw count to epg (see Table 1). A random sample from the Poisson distribution with a mean of 0.242 has a 2.5% probability of returning a value as high or higher than 2. This gives a lower 95% confidence interval of 0.242*50 or 12.1 epg. Likewise a random sample with a Poisson distribution of mean 7.22 has a 2.5% probability of returning a value as low or lower than 2. This gives an upper confidence interval of 7.22*50 or 361.2 epgs. Using this methodology, Table 1 gives some illustrative confidence limits of observed epgs given the dilution factor. This can readily be shown using the Poisson cumulative distribution function in Excel. The cumulative Poisson probability of 0.975 or lower confidence interval is shown by entering '=POISSON((count-1), 0.242,TRUE)' into a cell on the spreadsheet. Here the count is 2. The cumulative Poisson probabilities of 0.025 or the

upper confidence limit is shown by '=POISSON(count,7.22,TRUE)', again the count is 2.

Calculators for Poisson confidence intervals are available on the internet; for example GraphPad (www.graphpad.com/quickcalcs/ConfInterval1.cfm). Thus the raw untransformed count can be entered into this calculator which will give the confidence interval for this count. The untransformed count and the confidence intervals can then be multiplied by the dilution factor to give the estimate of the epg and its 95% confidence interval. Thus, whenever epgs are reported for individual animals it is very easy to report the 95% confidence interval of that epg.

Table 1. Observed untransformed egg counts seen on a McMaster slide and the resultant estimate of the epg with 95% confidence intervals given the appropriate dilution factor.

Untransformed egg count observed on the McMaster slide	Dilution factor	Calculated epg	Lower 95% confidence interval of epg	Upper 95% confidence interval of epg
0	50	0	0	184.5
1	50	50	1.3	278.6
2	50	100	12.1	361.2
5	50	250	81.2	583.4
10	50	500	239.8	919.5
20	50	1000	610.8	1544.4
0	10	0	0	36.9
1	10	10	0.3	55.7
2	10	20	2.4	72.2
5	10	50	16.2	116.7
10	10	100	48.0	183.9
20	10	200	122.2	308.9

Another inevitable conclusion can be drawn by the conformation with a Poisson distribution of raw egg counts on a McMaster slide. The variation of the estimated epg calculated by repeated samples from the same faecal sample decreases, as the numbers of samples taken to estimate that epg are increased. Indeed it can be predicted in a precise mathematical form. If repeated single samples are taken from a faecal sample with mean epg $n_1\mu_1$ then the coefficient of variation (standard deviation/mean) can be shown simply to be $1/\mathrm{sqrt}(\mu_1)$ and is not dependent on the dilution factor.

However if repeated multiple samples are made (eg 10 samples or 10 McMaster slides), you are effectively observing raw count data which has a Poisson distribution with a mean of the expected sum of the 10 observations (as the sum of independent Poisson variables is also Poisson). Consequently the coefficient of variation will vary with 1/sqrt(10* μ_1). Thus the coefficient of variation will be proportional to 1/sqrt(α) where α is the number of repeat samplings on the same faecal sample. This again can be illustrated with the 10 samples simulated earlier. This has a total count of 38 and pooling these count would give a confidence interval of 27 to 52 counts. The epg can now be calculated as 38 x 5 (as we have 10 repeat samples from the same faecal sample) or 190 as expected. The confidence limits of the of the epg can be shown to be 135 to 260 epg. If the simulation was repeated with just 5 random samples from a Poisson distribution of mean 4, this might generate the values 4, 1, 5, 7, 3. This gives a total count of 20 with an estimated epg of 20 x 10 or 200 epgs. The confidence limit (and hence coefficient of variation) is much wider at 122 to 309 epgs.

4. Results that inevitably arise due to Poisson processes.

With a knowledge of the Poisson process it is possible to replicate the data of some experiments studies quite easily with mathematics in a highly predictable manner. For example, Levecke et al., (2011) undertook a study to investigate if analytical sensitivity was important when monitoring drug efficacy against gastrointestinal nematodes when faecal egg counts are low. The study involved 30 calves, which had epgs of less than 200. From each calf 4 samples were examined by McMaster with an analytic sensitivity (or dilution factors) of 10,15, 33.3 and 50. A fifth sample was examined by the FLOTAC technique which has an analytic sensitivity of 1 (i.e. the dilution factor is 1). The mean epg of the 30 samples was then calculated and the number of zero counts recorded. The mean epgs were in the range of 60.5 to 75.6, with no zero counts being recorded for the FLOTAC and up to 11 zero counts for McMaster with an analytic sensitivity of 50. The epgs of individual calves were not recorded but a range given for each test. The FLOTAC technique had a minimum of 9 epg and a maximum of 160 epg and mean epg of 61.1. The following methodology can be used to demonstrate that the expected results for the McMaster examinations of Levecke et al. (2011) are entirely predictable with just the knowledge of the FLOTAC results. First it is possible to simulate the results of the 28 epgs for the calves not reported with individual FLOTAC results. This can be done using computer generated random numbers. In the example we report here we used a uniform random number generator which gives 28 samples. The mean of these 28 samples plus the additional samples of 9 and 160 reported (i.e. 30 samples in total) is constrained to a mean of 61.1. Alternatively 28 negative binomial distributed samples with mean 61.1 and a modest k of approximately 2.5 will suffice as Levecke et al. (2011) restricted their experiment to

non-zero counts below 200. This can then model variability between the calves. Each simulated epg is divided by the dilution factor to give a simulated raw count observed on the McMaster. For each simulated raw count, the expected proportion of zeros, using the Poisson distribution can be calculated and these summed over the 30 animals to obtain an expected number total number of zeros. The results are illustrated in table 2 (on this occasion for the generated data of uniformly distributed epgs) and compared to the actual results obtained by the various McMaster analytical sensitivities. Between animal negative binomial epgs give similar results (data not shown). What is clear is the predicted number of zeros conforms almost exactly to those observed in the study. Thus the answer to the question Levecke et al. (2011) posed "do the analytic sensitivity and formula matter?" is most certainly "yes"! However, you do not need to undertake a McMaster analysis of 120 samples to demonstrate this. But rather it can be easily shown by the application of the mathematics of Poisson processes.

Table 2. Observed data from Levecke et al. (2011) from a study of 30 calves with low faecal egg counts, together with the expected number of samples with zero counts calculated from the Poisson distribution, with between animal variation as described in the text. The expected mean of observed epg was calculated with FLOTAC with the various dilution factors (analytic sensitivity).

Analytic sensitivity (epg)	Mean observed (epg)	Min/ max epg	Number of samples with zero counts	Expected number of samples with zero counts calculated using the Poisson distribution.
1	61.1	9 to 160	0 out of 30	0 out of 30
10	64.7	0 to 230	3 out of 30	2 out of 30
15	60.5	0 to 150	4 out of 30	4 out of 30
33.3	75.7	0 to 399.6	8 out of 30	7 out of 30
50	71.5	0 to 350	11 out of 30	10 out of 30

Importantly and perhaps widely misunderstood is an observed zero count could still have come from a faecal sample that has a positive epg. This is due simply to the random sampling of the zero term in an appropriate Poisson distribution that has an mean >0. This is analogous to calculating exact binomial confidence intervals for disease prevalence in a sample where there are no diseased animals. The very real possibility, of an observed zero count actually coming from an animal with a considerable level of parasitism (Table 1), illustrates why zero counts cannot be ignored in any circumstance. Nevertheless Vidyashanker et al. (2012) (in the group discussion appendix) suggested ignoring zero counts when working with groups of horses numbering 50 or more. Furthermore, even with a high count of 1000 epg, using a dilution factor of 50 there is a 5% probability of over, or underestimating, the epgs by as much as 39.1% or 54.4%, respectively. In the same group

discussion appendix there was the suggestion that increasing the actual number of eggs being counted would reduce the variability in the counting process. This is true and consistent with variability introduced by Poisson processes. Nevertheless it was subsequently argued for a less stringent FECRT protocol because macrocytic lactone efficacies are high and further illustrates the lack of understanding of random variability inherent in FECs.

This also illustrates that the FLOTAC technique (Cringoli et al., 2010), where the dilution factor can be as low as 1 can reduce substantially the errors that inevitably occur when evaluating epgs. However, even with a analytical sensitivity of 1, with a low egg counts of say 1 or 2 epg, the diagnostic sensitivity will still only be 63% and 86% respectively because of Poisson processes.

5. Quality control

It is possible to introduce a simple quality control method when undertaking egg counts that can immediately identify excess variation or poor sampling from replicate counts. A fundamental property of the Poisson distribution is that the mean is equal to its variance. When taking small numbers of random samples from a Poisson distribution there can be quite large variations in the variance to mean ratio or Index of Dispersion (ID). How far the ID can depart from 1, whilst still being consistent with a Poisson distribution, depends on both the the mean of and numbers of replicates. A goodness of fit test is available in many statistical software packages and this could be used to test if replicate samples could have arisen from the same Poisson distribution. An alternative and relatively straightforward method is by examining the size of the ID. The ID is distributed as a $\chi 2$ distribution with n-1 (where n is the number of replicates) degrees of freedom providing the mean or number of replicates is sufficiently high (Selby, 1965). For low numbers of replicates and/or low expected mean there are departures from the $\chi 2$ distribution. Therefore, by using a Poisson random number generator, we have estimated the upper limits of ID using a simulation study by undertaking 10,000 simulations of series of replicates. From these simulated replicates we have calculated the upper 97.5 percentile of the distribution of the IDs. These are reported in Table 3 and can be used as a quick check that the variance to mean ratio of replicate samples is not too large. For example, if just two replicates were taken and these were 1 and 9, it gives a mean count of 5 and a variance of 32. The ID is 32/5 = 6.4. The maximum ID consistent with a Poisson distribution of mean 5 of two samples is 4.6. Indeed no two random samples from any Poisson distribution should have an ID greater than 5. Hence there is a problem with processing, mixing or counting of the sample and the replicates should be repeated. Similarly replicate counts of 1, 12 (ID =9.3); 2, 20 (ID=14); 68, 100 (ID=6.1) are unlikely to be Poisson distributed. However, replicate counts of 0, 2 (ID = 2); 2, 7 (ID=2.8) and 57, 74 (ID = 2.2) have sufficiently low ID to be consistent

with a Poisson distribution. With 2 replicates no samples with a mean greater than 10 should have an ID of greater than 5. Similarly with 5 replicates 2,5,4,7, 8 (mean =5.2, variance =5.7, ID =5.7/5.1=1.1) and 5 replicates of 20, 17, 14, 24, 30 (ID = 1.9) are consistent with a Poisson distribution whilst 0, 4, 6, 9, 11 (ID = 3.1) or 10,18,12, 28, 30 (ID=4.2) show statistically significant deviations from a Poisson distribution. For samples with greater number of replicates or higher sample means of the replicates, the maximum ID can be estimated from the appropriate $\chi 2$ distribution. If 20 replicates the ID could be no more than 1.73. This figure is derived from the the upper 2.5 percentile of the $\chi 2$ distribution with n-1 (20-1) or 19 degrees of freedom – 32.9. This is then divided by n-1, or 19 to arrive at 1.73. For 20 replicates, 1.73 is the limit regardless of the sample mean. Finally, if all the replicate counts are 0, there is no need to check the goodness of fit. Rather the upper CI of the epg can be estimated directly from the methods described above in section 3.

Table 3. Maximum index of dispersion (variance/mean) of the raw count data between replicates from the same faecal sample if the replicates are Poisson distributed.

Expected (mean) count of replicates	Number of replicates					
	2	3	4	5	7	10
1	3	3	2.9	2.4	2.3	2.1
2	4	3.5	2.9	2.7	2.3	2.1
3	4.5	3.5	3	2.7	2.4	2.1
5	4.6	3.5	3	2.7	2.4	2.1
7	4.8	3.6	3.1	2.7	2.4	2.1
≥10	5	3.7	3.1	2.8	2.4	2.1

6. Conclusions

The distribution of egg counts and parasites between different animals within a group is well known to be over-dispersed. For examples see Shaw and Dobson, (1995), Grenfell et al. (1995), Wilson et al. (1996) and Shaw et al. (1998). Likewise statistical methods that encompass this over-dispersion have been recommended to analyse anthelmintic efficacy (Torgerson et al., 2005). With the exceptions of Morgan et al. (2005), Dobson et al. (2009), El-Abdellati et al. (2010) and Schnyder et al. (2011) there appears to be little appreciation of the sampling errors that will inevitably arise from the use of faecal egg count techniques such as the McMaster, despite this being an extremely widely used diagnostic procedure. As illustrated in Table 1, the errors can be surprisingly large and cannot be overcome by further mixing the faecal suspension. Undertaking repeat counts on the same sample would help to ameliorate this problem. This latter option is effectively examining a larger mass of faeces and repeat counts should be Poisson distributed. Alternatively use of a

technique such as the FLOTAC which does not have a high dilution factor would reduce (but not eliminate) between count variability. Nevertheless, regardless of the quantitative technique used, the challenge remains to fully incorporate the diagnostic errors which inevitably arise from the Poisson process that occur in quantifying egg counts in faeces with the over-dispersed distributions that occur between separate animals and separate faecal samples.

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